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INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US96/14250	August 28, 1996	August 30, 1995

TITLE OF INVENTION: METHODS FOR LABELING DNA ENDS WITH HALOGENATED NUCLEOTIDES AND DETECTING SAME WITH ANTIBODIES

APPLICANT(S) FOR DO/EO/US: Zbigniew Darzynkiewicz, Xun Li and Frank Traganos

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [ ] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendemnts has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    Power of Attorney for All Designated States Except the U.S. (PCT Rule 90.4)
    Power of Attorney for U.S. National Prosecution (37 CFR Section 1.34)
    Declaration (37 CFR Sections 1.63 and 1.68)
    Verified Statement Claiming Small Entity Status - NonProfit Organization
    Response to Invitation to Correct Defects in the International Application

U.S APPLICATION NO (if known, see 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
Search Report has been prepared by the EPO or JPO	**$880.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$680.00**	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	~~$750.00~~ $770.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$1010.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	**$94.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =	$770.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	-0-	X $22.00	$	
Independent claims	5 - 3 =	2	X $80.00	$160.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $250.00	$	
	TOTAL OF ABOVE CALCULATIONS =			$ 930.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$ 465.00	
	SUBTOTAL =			$ 465.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
	TOTAL NATIONAL FEE =			$ 465.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00	
	TOTAL FEES ENCLOSED =			$505.00	
				Amount to be: refunded	$
				charged	$505.00

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-2385 (1075 PCT) in the amount of $505.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2385. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Robert S. MacWright, Esq.
Skadden, Arps, Slate, Meagher & Flom
919 Third Avenue
New York, New York 10022-3897

SIGNATURE:

Robert S. MacWright
NAME

32,425
REGISTRATION NUMBER

METHODS FOR LABELING DNA ENDS WITH HALOGENATED NUCLEOTIDES AND DETECTING SAME WITH ANTIBODIES

This invention was made with United States government support under grant R01 28704 awarded by the National Cancer Institute. The United States government has certain rights in the invention.

This application claims priority to U.S. Provisional Application Ser. No. 60/002,963, filed August 30, 1995, which is incorporated herein in its entirety by reference.

TECHINCAL FIELD

The present invention relates to the field of DNA detection for basic research, medical diagnostic testing, and forensic testing. More specifically, the invention relates to methods of detecting DNA by attaching labeling groups to the ends of DNA strands, and then detecting the labeling groups by using antibodies specific for those labeling groups to attach a secondary label that can be observed, for example, by eye or by a fluorescence or spectrophotometric detector.

The present invention has particular utility in detecting the occurrence of programmed cell death, which is known as "apoptosis." It also has utility as a method for detecting DNA replication and repair, and as a general method for DNA end labeling.

BACKGROUND ART

There is currently considerable interest in the process of programmed cell death, known as "apoptosis." This process is considered to be a normal part of an organism's biological defense system. For example, when cells become infected with certain viruses, the resulting overstimulation of the cellular machinery appears to trigger apoptosis; the infected cells die, and thus protect the

remaining cells from infection. It is also believed that apoptosis is an important weapon in the body's defense against cancerous growths, and that cancer results at least in part from a loss of the ability of affected cells to trigger their own death.

Researchers often wish to determine whether cells have died because of apoptosis, or because of some other cause. This is generally determined by examining the DNA of the cells. The DNA of cells that have died from apoptosis is typically broken into quite small and uniquely sized fragments, which is generally not the case when cells die from other causes. The DNA fragmentation that is characteristic of apoptosis is caused by enzymes in the cells known as endonucleases, and results in fragments of approximately 300 kilobases and 50 kilobases in length. Often, breaks in the DNA occur in sections between the nucleosome proteins, leading to broken pieces of DNA 180-200 base pairs long (Arends, Morris & Wyllie 1990, Compton 1992, Oberhammer *et al.* 1993, Walker *et al.* 1994, Wyllie, Kerr & Curie 1980).

To determine whether apoptosis has occurred, the DNA strand breaks need to be labeled in some manner so that they can be detected. Cell samples are generally first fixed with a crosslinking fixative, so that the small fragments of DNA are not lost from the sample cells in subsequent washing steps. The cells are then treated to make them permeable to further reagents. In two prior art methods, the permeabilized cells are then reacted with deoxynucleotides that have been labeled with either biotin or digoxygenin, using the enzymes DNA polymerase (the "nick translation" enzyme) or terminal deoxynucleotidyl transferase (TdT) to attach the labeled nucleotides to the 3'OH ends of the DNA fragments (Darzynkiewicz *et al.* 1992, Gorczyca *et al.* 1992; Gavrieli *et al.* 1992, Gorczyca, Gong & Darzynkiewicz 1993, Wijksman et al. 1993). The biotin or digoxygenin nucleotides themselves are not readily detectable; however, biotin can be specifically bound by the

lectin avidin, and antibodies that can specifically bind digoxygenin are also available. By binding avidin or digoxygenin antibodies to the fluorescent compound fluorescein, which can be done by reacting them with fluorescein-5-isothiocyanate (FITC), and then using these as secondary labels, the DNA strand breaks can be visualized by observing the fluorescence of the labeled DNA strand ends. A single step method utilizing deoxynucleotides directly labeled with fluorochromes, which is simpler but less sensitive than the above-described indirect methodology, has also been recently described (Gold *et al.* 1993, Li, *et al.* 1995).

Another area of active research is the study of DNA replication and repair. One method for detecting the cellular replication and/or repair of DNA is known as Strand Breaks Induced by Photolysis, or SBIP. In this method, the living cells are first supplied with halogenated DNA precursors such as BrdUrd or IdUrd, which the cells will incorporate into the DNA during replication or repair. The cells are then exposed to ultraviolet light, which causes the DNA to break where the halogenated precursors have been incorporated. The resulting DNA strand breaks can then be detected by fluorochrome labeled antibodies, as described above (Li, Traganos & Darzynkiewicz, 1994, Li *et al.* 1994c, 1995).

A third important use for DNA strand labeling is perhaps the oldest and most widely practiced; end-labeling of DNA strands during the purification and characterization of DNA, so that the DNA can be detected. As well appreciated by those in the field, DNA research samples are generally too small even to be seen with the naked eye. In order to detect them, they are often "end labeled" at a very early stage of purification. For example, it is common to end label DNA with nucleotides containing radioactive phosphorous (P^{32}); the presence of the DNA can then be detected at various stages of analysis using a geiger counter, by scintillation counting or by autoradiography.

However, such methods are currently being phased out due to the biological hazards involved in working with radioactive compounds, and because of the ecological problems caused by the need to dispose of such radioactive materials. These methods are being replaced largely by methods that provide for labeling with fluorescent compounds. However, methods in use prior to the present invention are often complex, and are generally somewhat expensive.

SUMMARY OF THE INVENTION

Each of the above-described prior art methods for DNA end labeling suffer from important limitations. The most important limitations are that the prior art methods are only moderately sensitive, and that they require the use of relatively expensive reagents. Such limitations are especially problematic when large numbers of DNA samples must be routinely analyzed, such as in medical diagnostic testing.

Upon reviewing the prices charged by vendors offering halogenated and fluorescein-labeled nucleotides, we noticed that the compound bromodeoxyuridine triphosphate, or "BrdUTP", costs only about one tenth of one percent as much as equivalent amounts of digoxygenin-, biotin-, or fluorochrome- conjugated deoxynucleotides. Our subsequent research showed that BrdUTP could be incorporated at the 3'OH ends of DNA at least as efficiently as directly labeled or digoxigenin or biotin labeled dUTP. We also discovered that strands of DNA into which the BrdUTP was incorporated could be readily detected by anti-BrdUrd antibodies which had been fluorescently labeled; anti-BrdUrd antibody is a widely available reagent, which is commonly used to detect the uptake of brominated uridine (BrdUrd) from culture media by living cells, as a measure of cell proliferation (Dolbeare & Selden 1994, Gratzner 1982). These two discoveries led to the present invention. The invention can be summarized as the enzymatic incorporation of halogenated deoxynucleotide triphosphates onto the 3'OH ends of DNA

strands, followed by the detection of the incorporated halogenated nucleotides using antibodies. Because of the low cost and general availability of reagents, this invention offers an attractive alternative to a variety of DNA end labeling methods currently in use. Surprisingly, this effective and inexpensive approach has heretofore been completely overlooked. We have also found that the sensitivity of BrdUTP detection is significantly higher than that of either biotin- or digoxygenin conjugated dUTP, which was quite unexpected. Of course, as will be readily appreciated by those of ordinary skill in the art, other halogenated deoxynucleotide triphosphates can be used in the present invention as well.

Accordingly, it is an object of the present invention to provide a simple and inexpensive method for the end-labeling of DNA strands.

It is a further object of the present invention to provide a method for the detection of DNA strands that is more sensitive than methods using either biotin or digoxygenin conjugated deoxynucleotides, and much more sensitive than direct labeling methods.

It is another object of the invention to provide an inexpensive yet sensitive method for the detection of apoptosis.

It is a further object of the invention to provide inexpensive yet sensitive methods for the detection of DNA replication and/or repair.

The objects of the invention that are recited above, as well as numerous other objects of the present invention, can be more readily understood from the following detailed description.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows isometric contour maps of DNA content vs. DNA strand break labeling for apoptotic cells, using a method according to the present invention (upper left panel), and also using four prior art methods;

Fig. 2 shows the results obtained by using the method of the present invention to detect strand breaks that were induced by ultraviolet irradiation of DNA containing BrdUrd incorporated during the process of DNA synthesis.

MODES FOR CARRYING OUT THE INVENTION

The methods of the present invention are quite straightforward both in design and in practice. Those of ordinary skill in the field of DNA labeling will readily appreciate that the primary requirements of the present invention are the use of a halogenated deoxynucleotide triphosphate, in conjunction with an enzyme that will attach the halogenated nucleotide to the 3'OH ends of DNA strands. For example, terminal deoxynucleotidyl transferase (TdT) or a DNA polymerase could be used for this purpose. Then, an antibody that specifically binds to the halogenated nucleotide, and which has itself been labeled with one or more detectable group, is added. The antibody binds to the brominated nucleotide, and the presence and/or location of the labeled antibody indicates the presence and/or location of the DNA.

In the examples provided herein, brominated deoxyuridine triphosphate (BrdUTP) has been used to label DNA strands by the methods of the present invention. However, as will be readily appreciated by those of ordinary skill in the field of DNA labeling, other halogenated deoxynucleotide triphosphates and corresponding antibodies can be substituted for BrdUTP and anti-BrdUrd antibodies. For example, bromo deoxyadenosine triphosphate (BrdATP) and anti-brominated adenosine antibodies may be used in the present method, as could iododeoxyuridine triphosphate (IdUTP) and anti-iodinated deoxyuridine antibodies. Various other nucleotide and antibody combinations can also be used, as will be appreciated by those in the field. Of course, it is important that the labeled antibodies used be specific for the particular halogenated nucleotide. Thus, it is expected, for example, that when iodinated uridine

triphosphate is attached to the 3'OH ends of the DNA strands, an antibody that specifically binds to iodouridine should be used. Of course, it is often possible to take advantage of cross-reactivity; some antibodies against halogenated nucleotides are known to show some cross-reactivity with nucleotides bearing a different halogen (Dolbeare & Selden 1994). For example, antibodies against iodinated deoxyuridine (IdUrd) can also react with brominated deoxyuridine (BrdUrd), and thus might be used to detect either one (although the affinity for IdUrd is higher).

In the examples given below, fluorescent monoclonal antibodies are used. However, the use of polyclonal antibodies is also within the scope of the present invention. In addition, it is not necessary that fluorescence be used as the detectable label. Those of ordinary skill in the art will readily appreciate that a wide spectrum of detectable labels have been used in biochemistry, and that any of them could be used in the present invention. For example, a radioactive moiety could alternatively be used, and such a moiety might either be contained within the structure of the nucleoside, or could be an added radioactive group. There are a variety of other detectable labels that can be attached to antibodies -- spin labels, chromophores, enzymes, enzyme-linked chromophoric systems, etc. -- and the use of such alternate labels would be well within the scope of the present invention.

The enzymatic addition of the halogenated deoxynucleotide in the methods of the present invention can be carried out using a variety of enzymes. The enzyme used must accept deoxynucleotide triphosphates and DNA strands as substrates; and must catalytically attach the deoxynucleotide to the DNA strand. As will be appreciated by those in the field, there are two well known enzymes that meet these requirements. One is terminal deoxynucleotide transferase (TdT), and the other is DNA polymerase. Various forms of these enzymes, for example,

from different source organisms, may be available, and all would be expected to be suitable for use in the present invention. However, it is not intended that the present invention be limited to the use of these enzymes, as any other enzyme having the aforementioned activity would also be suitable for use in the present invention. For example, there may be enzymes having that activity that are not presently known, but the use of those enzymes in the methods described herein would be well within the scope of the present invention.

It is expected that the methods of the present invention will generally result in the attachment of more than one halogenated nucleotide to each DNA strand. This multiple labeling provides certain advantages; it allows for the attachment of several antibody molecules per DNA strand, which we believe is at least in part responsible for the high sensitivity of the methods described. However, multiple labeling is not necessary to the present invention, and the addition of only a single halogenated nucleotide to each DNA strand and the subsequent detection of same using labeled antibodies is well within the scope of the present invention.

In the examples below, only a single halogenated nucleotide triphosphate is used. However, it would be well within the scope of the present invention to use a plurality of different halogenated nucleotide triphosphosphates in a single reaction, along with a corresponding plurality of labeled antibodies.

As demonstrated in the examples to follow, one advantage of the methods of the present invention is that they are more sensitive than many alternative methods. In many cell systems, DNA degradation during apoptosis is very extensive; in such cases, even the least sensitive methods of DNA strand break labeling may be adequate for detection of apoptotic cells. However, there may be situations when increased sensitivity is needed. For example, the present invention's high sensitivity will be of value in detecting

"atypical" apoptosis, which is when the endonucleolyic DNA cleavage stops at 50-300 kD size fragments rather than progressing to the production of smaller fragments (Chapman *et al.* 1995, Cohen *et al.* 1992, Collins *et al.* 1992, Oberhammer *et al.* 1993). The increased sensitivity of the present invention will also be of value when only very small samples are available for testing. In addition, the increased sensitivity of the present assay will be of value in labeling the relatively few primary DNA strand breaks that can be induced by ionizing radiation, certain antitumor drugs, free radicals, *etc.*, in order to estimate extent of DNA damage. Detecting the incorporation of BrdUrd during DNA repair may also require the more efficient labeling of DNA strand breaks that the present invention provides. Those of ordinary skill in the field will readily appreciate other situations in which the sensitivity of the present methods provides an advantage over alternative methods.

It is not necessary to the present invention that the reason for its superior sensitivity be understood. However, several possible explanations are apparent. One is that incorporation of BrdUTP by TdT is more efficient than incorporation of nucleotides which are conjugated to bulky fluorochromes or to digoxygenin/biotin. Another is that the detection of the incorporated BrdUrd by the antibody may be more efficient e.g. due to greater accessibility of the epitope. A third explanation may be that the number of FITC molecules attached to each molecule of antibody may be higher in the case of BrdUrd monoclonal antibodies than for fluorochrome conjugates.

As mentioned above, another major advantage of BrdUTP over other markers of DNA strand breaks is its low cost. Furthermore, BrdUrd antibodies are already widely used and are available from many sources (Dolbeare & Selden 1994, Gratzner, 1982). Use of BrdUTP for DNA strand break labeling, therefore, is expected to make analysis of apoptosis more widely available, especially in routine

clinical testing, when cost of the reagents plays a significant role in testing decisions. For example, preliminary clinical studies suggest that the analysis of tumor cell apoptosis during chemotherapy may provide evidence of drug effectiveness and be an early prognostic marker (Gorczyca *et al.* 1993a, Li *et al.* 1994a, Raza *et al.* 1995), and the availability of the inexpensive, simple methods of the present invention may make such testing more common.

The methods of the present invention can be best understood by reference to the following example experiments carried out using BrdUTP and using fluorescein labeled anti-brominated uridine antibodies for detection. Of course, it is not intended that these experiments limit the scope of the present invention, as many alternatives are possible, as described hereinabove and otherwise, as will be apparent to those of ordinary skill in the art.

GENERAL MATERIALS AND METHODS

Cells: HL-60 human promyelocytic leukemia cells were originally provided by Dr. Harry A. Crissman of the Los Alamos National Laboratory (Los Alamos, NM). The cells were maintained in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine (GIBCO) as previously described (Bruno *et al.* 1992, Bruno & Darzynkiewicz, 1992). The cells grew exponentially at densities below $5x10^5$ cells per ml.

Induction of Apoptosis: Apoptosis of HL-60 cells was triggered by cells' exposure to DNA topoisomerase I inhibitor camptothecin (CAM) which induces DNA lesions in form of the "cleavable complexes" (Hsiang *et al.* 1985). It was postulated that collision of DNA replicating forks with such lesions is responsible for cell death (Hsiang, Lihou & Liu, 1989). Indeed, apoptosis of HL-60 cells treated with CAM was shown to be very specific to cells progressing through S phase; cell death occurs rapidly and rather synchronously, affecting almost exclusively a population of

S phase cells (Fig. 1, see Del Bino *et al.* 1991). Exponentially growing HL-60 cells treated with CAM, thus, provide a convenient model to measure particular parameters of apoptotic cells. Populations of apoptotic cells as produced by this method are relatively uniform and can be compared, within the same sample, with the cell population not undergoing apoptosis i.e. with G_1 and G_2+M cells.

To induce apoptosis, the cells were incubated in the presence of 0. 15 μM DNA topoisomerase I inhibitor camptothecin (CAM; Sigma) for 3 hours, as described (Del Bino, Skierski & Darzynkiewicz 1991). For subsequent analysis, the cells were fixed in suspension in 1 % methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in Hanks' buffered salt solution (HBSS) for 15 min on ice, then centrifuged, rinsed with HBSS, resuspended in 70% ethanol. Such samples were stored at -20°C for up to four days.

Fluorescence measurements: Cellular fluorescence was measured using either a FASCan flow cytometer (Becton Dickinson) or the multiparameter Laser Scanning Cytometer (LSC; CompuCyte, Inc., Cambridge, MA, Kamentsky & Kamentsky, 199 1). Propidium iodide (PI) was added to each sample; this labels all double stranded DNA with a fluorescent red color, which can be distinguished from the green fluorescence of fluorescein, and serves as a control. The red (PI) and green (FITC, BODIPY) fluorescence from each cell were separated and quantified by FACScan using the standard optics and LYSYS 11 software (Becton Dickinson), as described (Li *et al.* 1995). Using the LSC, the cells were illuminated with 488 nm wavelength light from the argon ion laser, and the emissions were separated by a 570 nm dichroic mirror. The green (530±20 nm) and red (> 610 nm) fluorescence were measured by separate photomultipliers. Cells in G_2 phase were discriminated from cell doublets based on the difference in pulse shape. The instrument allows one to measure cells placed on slides, with rates up to 100 cells per sec, with an accuracy comparable

to that of flow cytometers (Kamentsky & Kamentsky 1991, Li *et al.* 1994c, 1995).

Controls: Control samples were analyzed similarly to the various test samples. Controls consisted of cells that were: (1) incubated in absence of BrdUrd; (2) incubated in absence of CAM to induce apoptosis; (3) incubated with the TdT reaction medium minus the TdT enzyme. All experiments were repeated at least 3 times, yielding essentially identical results.

EXAMPLE 1

DNA strand break labeling with BrdUTP: Cells were fixed by suspension in 1 % methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) in Hanks' buffered salt solution (HBSS) for 15 min on ice. The cells (approximately 10^6 per sample) were then rinsed twice with PBS and resuspended in 50 μ1 of TdT reaction buffer containing all the reagents as described above for the single step procedure, with the exception that instead of the fluorochrome-conjugated dUTP, 0.25 nmoles of BrdUTP (Sigma) was added. Following incubation (60 min at 37°C) the reaction was stopped by washing the cells twice in 15 mM Na_2EDTA-NaOH, pH 7.8. The cells were then incubated in 100 μl of a solution containing 0.7 μg of FITC-conjugated anti-BrdUrd MoAb (Becton Dickinson, San Jose, CA, clone B44), 0.1 % Triton X-100 (Sigma) and 1 % BSA. The cells were counterstained with 5 μg/ml of PI, in the presence of RNase, as described (Li *et al.* 1995). Samples were then analyzed by flow cytometry, as described above.

DNA strand break labeling using prior art methods:

One step (direct fluorescence) procedure: The direct procedure was primarily used to label DNA strand breaks in apoptotic cells. The fixed cells were rinsed twice with phosphate buffered salt solution (PBS) and incubated in 50 μl of TdT reaction buffer containing: 10 μl of 5 x concentrated buffer solution (I M potassium cacodyl-

ate; 125 mM Tris-HCI, pH 6.6; 1.26 mg/ml bovine serum albumin, BSA); 5 μl of 25 mM cobalt chloride; 0.5 μl (12.5 units) of TdT (all from Boehringer Mannheim, Indianapolis, IN) and 0.25 nmoles of fluorescinated dUTP (f-dUTP; from Boehringer) or fluoresceinated ATP (f-ATP; Boehringer Mannheim) or BODIPY® conjugated dUTP (B-dUTP; kindly provided by Dr. Richard P. Haugland, Molecular Probes). The volume of the incubation medium was adjusted with distilled water to 50 u1. The cells were incubated with the reaction buffer for 60 min at 37 C, then rinsed twice with 15 mM EDTA (pH 8.0) and once with 0. I% Triton X- 100 (Sigma) in PBS. The cells were then resuspended in 1 ml of PBS containing 2.5 μg/ml of propidium iodide (PI; Molecular Probes, Inc.) and 0. 1 % DNase free RNase (Sigma). Samples were analyzed by flow cytometry as described above.

Two-step procedure: This procedure was used to label both the photolysis-induced DNA strand breaks (SBIP) as well as DNA breaks in apoptotic cells. The samples were processed using the TdT (ApopagTag™) kit, kindly provided by ONCOR Inc. (Gaithersburg, MD), as described before (Li *et al.* 1994, 1995). Incorporation of digoxygenin conjugated dUTP (d-dUTP) into DNA by this kit is catalyzed by exogenous TdT; the incorporated d-dUTP is then detected with fluorescein labeled digoxygenin antibodies. In parallel experiments, DNA strand breaks were labeled using biotin labeled dUTP (b-dUTP) and fluoresceinated avidin (both from Boehringer Mannheim) as described before (Gorczyca *et al.* 1992, Li *et al.* 1993, 1994c). DNA was counterstained with 5 μg/ml of PI, in the presence of RNase, as described (Li *et al.* 1995). Samples were then analyzed by flow cytometry as described above.

Results

Fig. 1 shows bivariate distributions (isometric contour maps) of DNA content vs. DNA strand break labeling for exponentially growing HL-60 cells incubated for 3 h with 0.15 μM camptothecin which preferentially induces apoptosis

(Ap) of cells progressing through S phase (Del Bino *et al.* 1991). The first panel on the left, labeled "BrdUTP FITC-MoAb" shows the results obtained using a method of the present invention, wherein BrdUTP was incorporated at the 3'OH ends of DNA strands by terminal deoxynucleotidyl transferase (TdT), and the incorporated BrdUrd was detected by a fluorescently labeled BrdUrd monoclonal antibody. The arrow labeled "Ap" indicates the localization of cells that have undergone apoptosis. Note the exponential scale of the ordinate. The abscissa is relative DNA content per cell, based upon PI (red) fluorescence. The next two panels represent indirect labeling of DNA strand breaks utilizing prior art methods, wherein DNA strands are labeled by either digoxygenin conjugated dUTP (d-dUTP) or biotinylated dUTP (b-dUTP). The two right panels show cell distributions following a direct, single-step DNA strand break labeling, either with BODIPY or FITC conjugated dUTP; these are also prior art methods. As it is evident, the greatest separation of apoptotic from nonapoptotic cells is achieved following DNA strand break labeling with BrdUTP.

The results shown in Fig. 1 illustrate the differences in discrimination of apoptotic cells when the method of the present invention is used, as compared with several other methods of DNA strand break labeling: the indirect methods, utilizing b-dUTP or d-dUTP followed by FITC /avidin or FITC conjugated to digoxygenin MoAb (ApopTag kit manufactured by ONCOR), and the direct ones utilizing FITC- or BODIPY conjugated dUTP. The data demonstrate that although the direct methods of DNA strand break labeling employing f-dUTP or BODIPY-dUTP provided sufficient separation of the apoptotic from nonapoptotic cell populations (over eight-fold difference in fluorescence intensity), the DNA strand break associated fluorescence was significantly higher in the case of indirect labeling. Thus, the difference in fluorescence intensity between apoptotic and nonapoptotic (G_1) cells ("signal to noise ratio" in detecting apoptosis) was over 20- and over 37-fold for b-dUTP

(followed by FITC/avidin) and d-dUTP (followed by FITC/digoxygenin MoAb), which is over twice and over four-fold higher, respectively, compared to f-dUTP.

These quantitative differences between the various methods are further illustrated in Table 1. In this table, the relative fluorescence intensity of apoptotic cells is expressed as a ratio of the mean fluorescence intensity of the labeled apoptotic population to the mean fluorescence intensity of the nonapoptotic cells (Ap *vs.*G_1 cell populations). The mean values (+/- SD) of several (n = 4-10) estimates of this ratio for each method, obtained in separate experiments, are given. For comparison, the apoptosis labeling index represents the differences normalized with respect to the direct procedure employing FITC-dUTP.

Table 1.

Differences in fluorescence intensity of apoptotic cells following DNA strand break labeling with different methods.

Method, Fluorochrome	Relative Fluorescence Intensity	Apoptosis Labeling Index
Direct, FITC-dUTP	*8 .4 ± 0.4*	*1*
Direct, BODIPY-dUTP	*10.0 ± 1.7*	*1.2*
Indirect, biotin-dUTP FITC/avidin	*20.7 ± 3.4*	*2.5*
Indirect, digoxygenin-dUTP, FITC/ MoAb	*37.3 ± 2.1*	*4.4*
Indirect, BrdUTP FITC/MoAb	*72.0 ± 15.3*	*8.6*

Surprisingly, the difference in intensity of DNA strand break labeling-associated fluorescence between apoptotic and nonapoptotic cell populations was highest in the case of labeling with BrdUTP. The "signal to noise ratio" provided by this method of DNA strand break labeling

was over 70-fold, which was more than eight-fold better compared with the direct labeling methods and nearly twice as good as that offered by the most sensitive indirect assay, namely the one based on d-dUTP incorporation.

EXAMPLE 2

Induction of DNA strand breaks in BrdUrd-labeled cells by UV illumination (SBIP): To label DNA replicating cells, BrdUrd (Sigma Chemical Co., St. Louis MO) was added to cultures at a concentration of 20 μM, for 40 min. For analysis of BrdUrd incorporation using the SBIP method, the cells incubated with this precursor were treated with 2% dimethyl sulfoxide (DMSO; Sigma) and 20 μg/ml of Hoechst 33258 (Molecular Probes, Inc., Eugene, OR) for additional 20 min. The cells were then centrifuged, resuspended in 2 ml of ice cold HBSS, and the cell suspensions were transferred into 60 x 15 mm polystyrene Petri dishes (Corning, N.Y.). The dishes were then placed directly on the glass surface of a Fotodyne UV 300 analytic DNA transilluminator, containing four 15 watt bulbs (Fotodyne Inc., New Berlin, WI) providing maximal illumination at 300 nm wavelength, as described before (Li *et al.* 1994b, 1994c. 1995). The cells were exposed to UV light for 5 min; exposure to UV light photolyses the DNA; i.e., induces DNA strand breaks at the sites of BrdUrd incorporation (Li et al., 1994(c)). The average intensity of UV light at the surface on which the cells were exposed, measured by a UVX-25 sensor (UVP, Inc.,Upland, CA), was 4.5 mW/cm^2. The cells were then centrifuged, suspended in HBSS, fixed in suspension in 100% methanol on ice, and stored in methanol at minus 20°C for up to four days.

Labeling of DNA Strand Breaks with BrdUTP: DNA strand breaks were then labeled with BrdUTP followed by labeling with anti-BrdUrd monoclonal antibody conjugated to FITC, as described in Example 1 above. PI was also added to label DNA as a measure of DNA content, as described above. The treated cells were then either measured by flow cytome-

try or by the Laser Scanning Cytometer (LSC, CompuCyte, Inc.), as also described above.

Results

The results are shown in Fig. 2. The two left panels show the results obtained using flow cytometry; the two right panels show the results obtained using the Laser Scanning Cytometer. Both methods gave acceptable results. The two bottom panels represent cells that were not exposed to UV light; note that apparently no break sites were introduced. It should be noted that without the step of DNA denaturation (which requires cell treatment with strong acids or heating; Dolbeare *et al.* 1983, Dolbeare & Selden, 1994), BrdUrd incorporated during DNA replication was undetected by the monoclonal antibody used. This was evident from the fact that no labeling was observed when the photolysis step was omitted (Fig. 2, bottom panels).

INDUSTRIAL APPLICABILITY

The present invention has industrial applicability in the fields of scientific research, diagnostic medicine, forensic medicine. More specifically, the invention can be used by researchers to label DNA in the course of, for example, genetic research. It can also be used by diagnostic laboratories wishing to test patient samples, for example, to determine if cells have undergone apoptosis. The invention can also be used in forensic medicine, for example, to end label DNA fragments used in restriction fragment lenght polymorphism (RFLP) analysis to determine the origin of crime scene samples. A wide spectrum of other industrial applications will be readily apparent to those of skill in the art.

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We Claim:

1. A method for labeling DNA strands, comprising the steps of:
   a. incubating said DNA strands with a halogenated deoxynucleotide triphosphate (HdNTP) and an enzyme that catalytically attaches the halogenated deoxynucleotide (HdN) of said HdNTP onto the 3'OH ends of said DNA strands; and
   b. reacting the resulting HdN-DNA strands with a labeled anti-halogenated deoxynucleotide (anti-HdN) antibody which specifically binds to said HdN.

2. The method of Claim 1 wherein said halogenated deoxynucleotide triphosphate is selected from the group consisting of brominated deoxyadenosine triphosphate; brominated deoxycytosine triphosphate; brominated deoxyguanosine triphosphate; brominated deoxyuridine triphosphate; brominated deoxythymidine triphosphate; iodinated deoxyadenosine triphosphate; iodinated deoxycytosine triphosphate; iodinated deoxyguanosine triphosphate; iodinated deoxyuridine triphosphate; and iodinated deoxythymidine triphosphate.

3. The method of Claim 1 wherein said enzyme is selected from the group consisting of terminal deoxynucleotidyl transferase (TdT) and DNA polymerase.

4. The method of Claim 1 wherein said labeled anti-halogenated nucleotide (anti-HdN) antibody is selected from the group of fluorescently labeled anti-HdN monoclonal antibody; radiolabeled anti-HdN monoclonal antibody; peroxidase-labeled anti-HdN monoclonal antibody; chromophore labeled anti-HdN monoclonal antibody; fluorescently labeled anti-HdN polyclonal antibody; radiolabeled anti-HdN polyclonal antibody; peroxidase-labeled anti-HdN

polyclonal antibody; and chromophore labeled anti-HdN polyclonal antibody.

5. A method for labeling DNA strands, comprising the steps of:
   a. incubating said DNA strands with brominated deoxynucleotide triphosphate (BrdNTP) and an enzyme that catalytically attaches the brominated deoxynucleotide (BrdN) of said BrdNTP onto the 3'OH ends of said DNA strands; and
   b. reacting the resulting BrdN-DNA strands with a labeled anti-brominated deoxynucleotide (anti-BrdN) antibody which specifically binds to said BrdN.

6. The method of Claim 5 wherein said brominated deoxynucleotide triphosphate is selected from the group consisting of brominated deoxyadenosine triphosphate; brominated deoxycytosine triphosphate; brominated deoxyguanosine triphosphate; brominated deoxyuridine triphosphate; and brominated deoxythymidine triphosphate.

7. The method of Claim 5 wherein said enzyme is selected from the group consisting of terminal deoxynucleotidyl transferase (TdT) and DNA polymerase.

8. The method of Claim 5 wherein said labeled anti-brominated nucleotide (anti-BrdN) antibody is selected from the group of fluorescently labeled anti-BrdN monoclonal antibody; radiolabeled anti-BrdN monoclonal antibody; peroxidase-labeled anti-BrdN monoclonal antibody; chromophore labeled anti-BrdN monoclonal antibody; fluorescently labeled anti-BrdN polyclonal antibody; radiolabeled anti-BrdN polyclonal antibody; peroxidase-labeled anti-BrdN polyclonal antibody; and chromophore labeled anti-BrdN polyclonal antibody.

9. A method for labeling DNA strands, comprising the steps of:

   a. incubating said DNA strands with brominated deoxyuridine triphosphate (BrdUTP) and an enzyme that catalytically attaches the brominated uridine (BrdUrd) of said BrdUTP onto the 3'OH ends of said DNA strands; and

   b. reacting the resulting BrdUrd-DNA strands with a labeled anti-brominated uridine (anti-BrdUrd) antibody which specifically binds to said BrdUrd.

10. The method of Claim 9 wherein said enzyme is selected from the group consisting of terminal deoxynucleotidyl transferase (TdT) and DNA polymerase.

11. The method of Claim 9 wherein said anti-brominated uridine (anti-BrdUrd) antibody is selected from the group of fluorescently labeled anti-BrdUrd monoclonal antibody; radiolabeled anti-BrdUrd monoclonal antibody; peroxidase-labeled anti-BrdUrd monoclonal antibody; chromophore labeled anti-BrdUrd monoclonal antibody; fluorescently labeled anti-BrdUrd polyclonal antibody; radiolabeled anti-BrdUrd polyclonal antibody; peroxidase-labeled anti-BrdUrd polyclonal antibody; and chromophore labeled anti-BrdUrd polyclonal antibody.

12. A method for detecting breaks in DNA strands, comprising the steps of:

   a. incubating said DNA strands with brominated deoxyuridine triphosphate (BrdUTP) and an enzyme that catalytically attaches the brominated uridine (BrdUrd) of said BrdUTP onto the 3'OH ends of said DNA strands;

b. reacting the resulting BrdUrd-DNA strands with a labeled anti-brominated uridine (anti-BrdUrd) antibody which specifically binds to said BrdUrd; and

c. detecting said label.

13. The method of Claim 12 wherein said enzyme is selected from the group consisting of terminal deoxynucleotidyl transferase (TdT) and DNA polymerase.

14. The method of Claim 12 wherein said labeled anti-brominated uridine (anti-BrdUrd) antibody is selected from the group of fluorescently labeled anti-BrdUrd monoclonal antibody; radiolabeled anti-BrdUrd monoclonal antibody; peroxidase-labeled anti-BrdUrd monoclonal antibody; chromophore labeled anti-BrdUrd monoclonal antibody; fluorescently labeled anti-BrdUrd polyclonal antibody; radiolabeled anti-BrdUrd polyclonal antibody; peroxidase-labeled anti-BrdUrd polyclonal antibody; and chromophore labeled anti-BrdUrd polyclonal antibody.

15. The method of Claim 12 wherein said labeled anti-brominated uridine (anti-BrdUrd) antibody is selected from the group of fluorescently labeled anti-BrdUrd monoclonal antibody and fluorescently labeled anti-BrdUrd polyclonal antibody, and said detecting is accomplished by a method selected from the group of flow cytometery, fluorescence microscopy, multiparameter laser scanning microscopy, and visual observation during irradiation with light of the excitation wavelength.

16. The method of Claim 12 wherein said labeled anti-brominated uridine (anti-BrdUrd) antibody is selected from the group of radiolabeled anti-BrdUrd monoclonal antibody and radiolabeled anti-BrdUrd polyclonal antibody, and said detecting is accomplished by a method

selected from the group of scintillation counting, autoradiography, and geiger counting.

17. A method for detecting whether cells have undergone apoptosis, comprising the steps of:
    a. Fixing said cells;
    b. incubating said cells with brominated deoxyuridine triphosphate (BrdUTP) and an enzyme that catalytically attaches the brominated uridine (BrdUrd) of said BrdUTP onto the 3'OH ends of DNA strands in said cells;
    c. reacting the resulting BrdUrd-DNA strands with a labeled anti-brominated uridine (anti-BrdUrd) antibody which specifically binds to said BrdUrd; and
    d. detecting said label, wherein apoptosis is confirmed by the detection of label at a level more than about two standard deviations above the mean level of label found in identically treated contol samples known not to have undergone apoptosis.

18. The method of Claim 17 wherein said enzyme is selected from the group consisting of terminal deoxynucleotidyl transferase (TdT) and DNA polymerase.

19. The method of Claim 17 wherein said labeled anti-brominated uridine (anti-BrdUrd) antibody is selected from the group of fluorescently labeled anti-BrdUrd monoclonal antibody; radiolabeled anti-BrdUrd monoclonal antibody; peroxidase-labeled anti-BrdUrd monoclonal antibody; chromophore labeled anti-BrdUrd monoclonal antibody; fluorescently labeled anti-BrdUrd polyclonal antibody; radiolabeled anti-BrdUrd polyclonal antibody; peroxidase-labeled anti-BrdUrd polyclonal antibody; and chromophore labeled anti-BrdUrd polyclonal antibody.

20. The method of Claim 17 wherein said labeled anti-brominated uridine (anti-BrdUrd) antibody is selected

from the group of fluorescently labeled anti-BrdUrd monoclonal antibody and fluorescently labeled anti-BrdUrd polyclonal antibody, and said detecting is accomplished by a method selected from the group of flow cytometery, fluorescence microscopy, multiparameter laser scanning microscopy, and visual observation during irradiation with light of the excitation wavelength.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application:	METHODS FOR LABELING DNA ENDS WITH HALOGENATED NUCLEOTIDES AND DETECTING SAME WITH ANTIBODIES
Inventors:	Zbigniew Darzynkiewicz, Xun Li and Frank Traganos
Ser. No.:	PCT/US96/14250
Filing Date:	August 28, 1996
Priority Date:	August 30, 1995
Priority To:	Provisional Application 60/002,963
Examiner:	Not yet assigned
Art Unit:	Not yet assigned
Assignee:	New York Medical College
Attorney:	Robert S. MacWright (212)735-2121
Reg. No:	32,425
Client/Matter:	001480/0003
File Reference:	1075PCT

Action:	**VERIFIED STATEMENT CLAIMING SMALL ENTITY STATUS, NONPROFIT ORGANIZATION**
Rules:	37 C.F.R. §§ 1.27(d), 1.9(f), 1.9(e)(1)

Commissioner of Patents and Trademarks
Washington, DC 20231

Dear Sir:

1. I hereby declare that:

a. I am an official empowered to act on behalf of New York Medical College, which has a principal place of business at its Office of Research Administration, Administration Building Room 202, Grasslands Road, Valhalla, NY 10595 US.

b. New York Medical College is the assignee of the entire interest in the above-described PCT application, the inventions and discoveries described therein, and of all National Phase applications arising therefrom, including the corresponding National Phase application in the United States.

c. New York Medical College is a nonprofit organization as defined in 37 C.F.R.§ 1.9(e)(1) ("a university or other institution of higher education located in any country") for the purposes of paying reduced fees to the United States

Skadden, Arps, Slate, Meagher & Flom
919 Third Ave., New York, NY 10022 (212)735-3000

Patent and Trademark Office upon filing and during prosecution of the U.S. National Phase application corresponding to the PCT application identified above.

d. All rights under contract or law to the above-described U.S. National Phase application, and the inventions and discoveries described therein, have been conveyed to and remain with New York Medical College; and no other person, concern, or organization has rights in the applications or the inventions or discoveries described therein.

2. I acknowledge the duty to file in the above-described U.S. National Phase application a notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 C.F.R. §1.28(b)).

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements and the like may jeopardize the validity of the U.S. National Phase application or any patent issued thereon.

Signature: Catharine Crea

Name: **Catharine Crea**

Title: **Assistant Dean**

Date: October 15, 1996

Skadden, Arps, Slate, Meagher & Flom
919 Third Ave., New York, NY 10022 (212)735-3000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application:	METHODS FOR LABELING DNA ENDS WITH HALOGENATED NUCLEOTIDES AND DETECTING SAME WITH ANTIBODIES
Inventors:	Zbigniew Darzynkiewicz, Xun Li and Frank Traganos
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Priority Date:	August 30, 1995
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Examiner:	Not yet assigned
Art Unit:	Not yet assigned
Assignee:	New York Medical College
Attorney:	Robert S. MacWright (212)735-2121
Reg. No:	32,425
Client/Matter:	001480/0003
File Reference:	1075PCT

Action:	**DECLARATION**
Rules:	37 C.F.R. §§ 1.63, 1.68

Commissioner of Patents and Trademarks
Washington, DC 20231

Dear Sir:

We the undersigned hereby declare as follows:

1. This declaration is directed to the patent specification that is described in the heading above.

2. Our respective residences, post office addresses and countries of citizenship are as set forth under our signatures below.

3. We are joint inventors of the invention claimed in the above-described specification.

4. We have each reviewed and understand the contents of the specification, including the claims.

5. We believe that we are the original and first inventors of the subject matter which is claimed and for which a patent is sought.

6. We hereby acknowledge our duty to disclose to the Patent and Trademark Office all information known to either of us to be material to patentability, as defined in 37 C.F.R. § 1.56.

7. This application claims priority to provisional application 60/002,963, which was filed on August 30, 1995 (which was less than one year prior to the filing date of the instant application).

8. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements and the like may jeopardize the validity of the application or any patent issued thereon.

Signature: Zbigniew Darzynkiewicz

1-00

Name: Zbigniew Darzynkiewicz
Residence: Chappaqua, New York, United States
Post Office Address: 37 Meadow Lane
Chappaqua, NY 10514 US NY
Citizenship: US
Date: October 15, 1996

Signature: Xun Li

2-00

Name: Xun Li
Residence: Yorktown Heights, New York, United States
Post Office Address: 1635 Morningview Drive
Yorktown Heights, NY 10598 US NY
Citizenship: China
Date: October 15, 1996

Signature: Frank Traganos

3-00

Name: Frank Traganos
Residence: New York, New York, United States
Post Office Address: 301 66th Street
New York, NY 10021 US NY
Citizenship: US
Date: October 15, 1996

Skadden, Arps, Slate, Meagher & Flom
919 Third Ave., New York, NY 10022 (212)735-3000

DNA break labelling

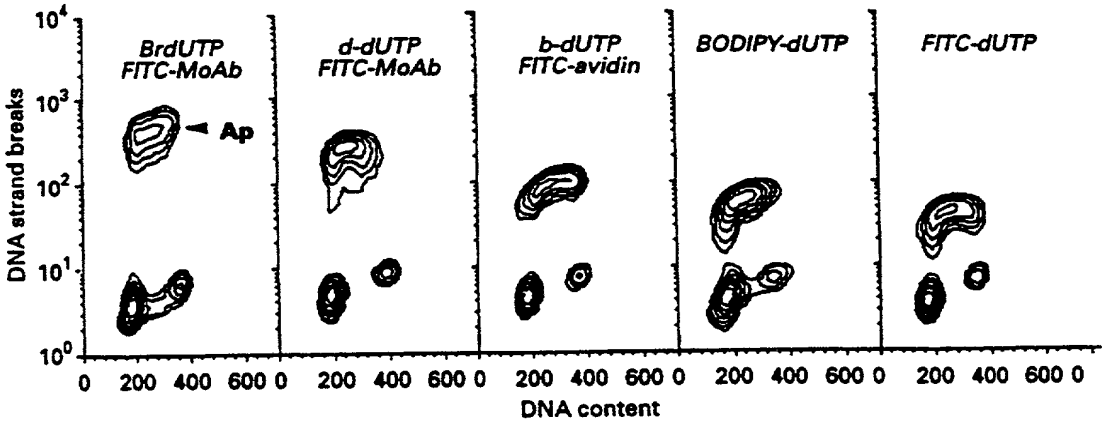

Fig. 1

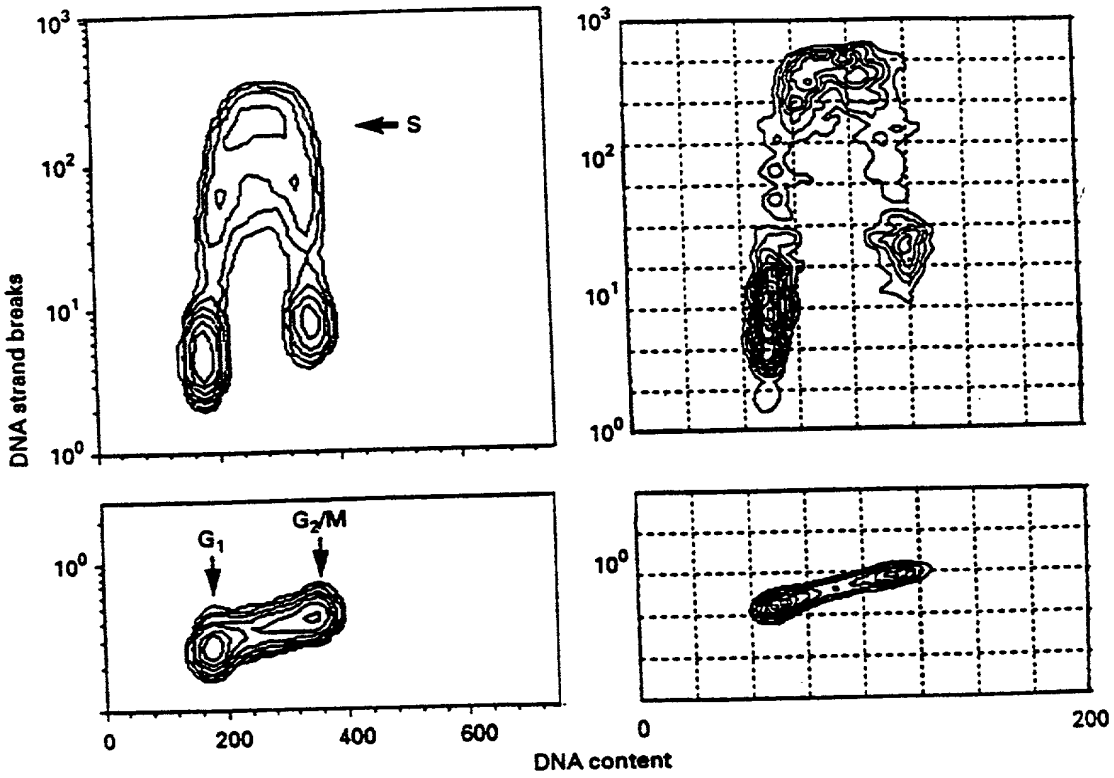
DNA strand breaks
10^3
10^2
10^1
10^0
S
G_1
G_2/M
0
200
400
600
DNA content

Fig. 2